IN THE U.S. PATENT AND TRADEMARK OFFICE

In re application of		Before the Board of Appeals	
Yuji HATADA et al.		Appeal No.:	
Appl. No.:	08/952,741	Group:	1652
Filed:	November 25, 1997	Examiner:	Slobodyansky, E.
Conf.:	3031		
For:	GENE ENCODING ALKALINE LIQUIFYING ALPHA-AMYLASE		

APPEAL BRIEF TRANSMITTAL FORM

Assistant Commissioner for Patents
Washington, D.C. 20231:

May 13, 2002

Sir:

Transmitted herewith is an Appeal Brief (in triplicate) on behalf of the Appellants in connection with the above-identified application.

☐ The enclosed document is being transmitted via the Certificate of Mailing provisions of 37 C.F.R. 1.8.

A Notice of Appeal was filed on May 31, 2001.

☐ Applicant claims small entity status in accordance with 37 C.F.R. § 1.27

The fee has been calculated as shown below:

☒ Extension of time fee pursuant to 37 C.F.R. §§ 1.17 and 1.136(a) - $920.00 (large entity)

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Respectfully submitted,

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APPEAL BRIEF

Assistant Commissioner for Patents
Washington, DC 20231

May 13, 2002

Sir:

In response to the Examiner's Office Actions dated June 7, 2001, December 4, 2000, and May 24, 2000, and in response to several conversations with the Examiner concluding with the conversation of March 26, 2002, the following Appeal Brief is respectfully submitted in connection with the above-identified application.

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I. Real Party in Interest

The real party in interest of the present invention is Kao Corporation of Tokyo, Japan, the assignee of the entire right and interest of the instant application. The assignment of said right and interest was recorded on November 25, 1997 at Reel 9071, Frame 0888.

II. Related Appeals and Interferences

There are no related appeals or interferences pending for the present application.

III. Status of Claims

Claims 2-7, 12, 13, 15, 16 and 20-24 are pending in the present application. The Examiner has indicated in the Advisory Action of June 7, 2001 that claims 2 and 5-7 would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claims and rewriting claims 2 and 5-7 into independent form. As stated in the Advisory Action of April 2, 2002, the Examiner entered the amendment filed March 12, 2002; claims 2, 5-7 and 12 and 13 are allowed.

The standing rejections of claims 3, 4, 15, 16, and 20-24 are appealed. Claims 3, 4, 15, 16, and 20-24 are rejected under 35 USC §112, first paragraph and claims 3, 4, 15, 16, and 20-24 are

rejected under 35 USC §103(a) as being unpatentable over Ara '367 (EP 0 670 367 A1) in view of Tsukamoto et al. (Biochem. Biophys. Res. Comm., 151(1), pp. 25-31, (1988)) or Yuuki et al. (J. Biochem., 98(5), pp. 1147-1156, (1985)).

IV. Status of Amendments

Subsequent to the Examiner's final rejection of claims 2-7, 14-16 and 20-24 on December 4, 2000, after-final responses were filed May 31, 2001 and February 12, 2002, attempting to amend claims 2, 3, 20, 22, 23, and 24, but the Examiner denied entry of the amendments. The Examiner indicated in a conversation on March 26, 2002 that the amendment of March 12, 2002 would be entered and an Advisory Action was mailed April 2, 2002 confirming entry of that Amendment and allowability of claims 2, 5-7, 12, and 13. A Notice of Appeal was filed with the after-final response on May 31, 2001. Claims 3, 4, 15, 16, and 20-24 are appealed.

V. Summary of Invention

The present invention provides a DNA fragment encoding alkaline liquefying α-amylase protein, having maximal activity at a pH of 8-9, and such alkaline liquefying α-amylase proteins in which one or more amino acids of a specified amino acid sequence are substituted, added, deleted, or inserted, while retaining its

enzymatic activity. (See page 11, lines 19-20 and page 11, line 24 to page 12, line 2). Further, the instant invention provides recombinant DNA containing the DNA fragment encoding alkaline liquefying α-amylase, a transformed microorganism harboring the recombinant DNA, as well as a method for producing alkaline liquefying α-amylase using the transformant, and proteins in which one or more amino acids are substituted, added, deleted, or inserted. (See page 5, lines 8-20, and page 11, lines 7-19). The method of the present invention enables mass production of alkaline liquefying α-amylases useful as a detergent component. (See page 19, lines 6-11).

VI. Issues to be considered

Issue 1

Does the specification, taken with what was known by one of skill in the art at the time of filing the present application, provide adequate written description for claims 3, 4, 15, 16 and 20?

That is, is there adequate written description for:

(a) the structural language "a DNA encoding an α-amylase having an amino acid sequence of SEQ ID NO: 2 with one or more amino acids substituted, added, deleted or inserted" and

(b) the functional language "without changing enzymological properties", having maximal activity "at a pH optimum of 8-

9", and "having the ability to hydrolyze 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) without hydrolyzing pullulan"?

Issue 2

Does the specification, taken with what was known by one of skill in the art at the time of filing the present application, provide adequate written description of the following functional elements recited in claim 21:

activity in a stated pH range;

a stated degree of stability in a stated pH;

activity in a stated temperature range of 20°C to 80°C, with a stated optimum temperature range;

stability in the face of a recited treatment;

a selected molecular weight;

a stated isoelectric point;

stability in the presence of certain cations; and

freedom from inhibition of enzyme activity by certain chemicals?

Issue 3

Does the specification, taken with what was known by one of skill in the art at the time of filing the present application, provide adequate written description of claims 22-24, which recite the

structural elements provided by SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11 and the functional element of "encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9"?

Issue 4

Does the specification, taken with what was known by one of skill in the art at the time of filing the present application, enable the skilled artisan to make and use "an α-amylase having an amino acid sequence of SEQ ID NO:2 with one or more amino acids substituted, added, deleted or inserted" and having certain recited functional properties?

Issue 5

Does the specification, taken with what was known by one of skill in the art at the time of filing the present application, enable the skilled artisan to make and use an isolated DNA comprising structural elements provided by SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 11 and "encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9"?

Issue 6

Are claims 3, 4, 16, and 20-24 unpatentable over Ara '367 in view of Tsukamoto et al. or Yuuki et al.?

VI. Grouping of Claims

Appellants respectfully request that the claims be grouped as follows.

Group I - claims 3, 4, 15, 16, and 20

Group II - claim 21

Group III - claims 22-24

Each group of claims raises different issues for consideration by the honorable Board of Patent Appeals and Interferences as follows:

Group I -Issues 1, 4, and 6

Group II - Issues 1, 2, 4, and 6

Group III - Issues 3, 5, and 6

VII. Arguments

Issue 1

The Specification provides adequate written description of

(a) the structural language "a DNA encoding an α-amylase having an amino acid sequence of SEQ ID NO: 2 with one or more amino acids substituted, added, deleted or inserted" and

(b) the functional language "without changing enzymological properties", having maximal activity "at a pH optimum of 8-9", and having the ability to hydrolyze "1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation

products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6)" without hydrolyzing pullulan as well as other functional elements for claims dependent from claim 3.

Applicants assert that claims 3, 4, 15, 16, and 20 are not properly rejected under 35 USC §112, first paragraph for lacking description of a DNA encoding an α-amylase having an amino acid sequence of SEQ ID NO: 2 with one or more amino acids substituted, added, deleted or inserted and further having certain functional properties.

Independent claim 3 reads as follows:

> *3. A DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9 and possessing an amino acid sequence which has been obtained by modifying an amino acid sequence described in SEQ ID NO:2 in a manner* ***in which one or more amino acids are substituted, deleted, or inserted*** *without changing enzymological properties of said amino acid sequence described in SEQ ID NO:2 and hydrolyzes 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) and does not hydrolyze pullulan.*

Issue 1 relates to the words "in which one or more amino acids are substituted, deleted, or inserted". The Examiner stated in the Office Action of December 4, 2000:

> *Claim 3 encompasses a great number of α-amylases having unknown structures and possessing the requisite properties. The Examiner is unable to locate adequate support in the*

specification for such α-amylases. Thus there is no indication that α-amylases having amino acid sequences other than SEQ ID NO: 2 and having the requisite properties were within the scope of the invention as conceived by Applicants at the time the application was filed.

As an initial matter, Applicants wish to point out that the literal language "in which one or more amino acids are substituted, deleted, or inserted" occurs on page 12, lines 1-3. This indicates that Applicants considered variants of SEQ ID NO: 2 in which the sequence is modified by having one or more amino acids substituted, deleted, or inserted as within the scope of the invention at the time the application was filed.

At issue is whether Applicants had possession of the full-scope of the claimed invention at the time of filing the application. Applicants assert that they did have possession of the full scope of the claimed invention (for the claims at issue) for the following reasons.

The "Revised Interim Written Description Guidelines Training Materials" (http://www.uspto.gov/web/offices/pac/writtendesc.pdf visited on September 5, 2001) issued by the U.S. Patent and Trademark Office partly in response to the case *University of California v. Eli Lilly*, 43 USPQ2d 1398 (Fed. Cir. 1997) provides examples of what constitutes adequate written description of a cloned DNA invention. On page 53 of the "Revised Interim Written Description Guidelines Training Materials" (hereinafter, written

description training materials) appears an example of a product described by function that is analyzed and found to have adequate written description. The claim recited in this example says

> *A protein having SEQ ID NO: 3 and variants thereof that are at least 95% identical to SEQ ID NO: 3 and catalyze [SIC] the reaction of A→B.*

Similar to this claim, the instant claim 3 recites a sequence (i.e. SEQ ID NO:2) and variants thereof (*i.e.* in which one or more amino acids are substituted, deleted, or inserted without changing enzymological properties of said amino acid sequence). Further, the instant claim 3 has language equivalent to the phrase, "catalyze the reaction of A→B". That is, claim 3 recites

> *and hydrolyzes 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6).*

The Example in the written description training materials indicates that any enzyme in the genus "catalyzes the reaction of A→B". It does not say how well or the rate at which it must catalyze the reaction from A→B. Thus, the Example provides a less stringent limitation than that enumerated in the instant claim 3, which recites a defined level of activity, i.e., "*without changing enzymological properties of said amino acid sequence described in SEQ ID NO:2*". Therefore, claim 3 is in this sense narrower than the "safe harbor" example in the written description training materials.

The analysis in the Written Description Training materials further indicates that only one species was presented in the disclosure. This is similar to the instant invention wherein Applicants have provided one actual species and one hypothetical species. (See page 12, lines 3-6).

The example provided in the written description training materials also has a limitation in it that says the enzyme variants must have 95% sequence identity to the enzyme having the sequence given by SEQ ID NO: 3. The instant claim 3 does not have a limitation that limits the variants to 95% identity in sequence. However, there are three other elements in claim 3 that definitively describe the genus. The first element is that the generated mutant enzyme must have optimal activity at pH 8-9, the second element is that the enzymological properties are the same as those of an enzyme of SEQ ID NO: 2, and the third element is that the mutant enzyme does not cleave pullulan.

When this is combined with what is known in the art about amylases, Applicants have provided a well-defined genus. The prior art teaches that amylases are known to have four regions, designated regions I-IV, which are highly conserved (see page 13, last line to page 14, line 3).[1] One of skill in the art would recognize that these are regions wherein, if any changes were made

at all, one would at most make conservative amino acid substitutions. One or more amino acids would not likely be inserted or deleted into these regions. Applicants contend that this knowledge to the art provides the practitioner with description of structural constraints similar to what is achieved by the concept of 95% sequence homology presented in the example from the written description training materials.

Furthermore, in the example from the training materials, there is no indication if any other enzymes similar to the enzyme from liver being claimed are known. If similar enzymes are not known, then one of skill in the art would not know where to make amino acid substitutions, deletions, or additions and still retain the activity of any mutant enzyme. This is opposite of the case of amylases. Because many amylases are known in the art, one of skill in the art has a starting point from which to direct amino acid substitutions, deletions, or additions. Further still, claim 3 includes functional limitations as noted above.

When these functional limitations are combined with the structural limitation of variation of SEQ ID NO: 2, a genus is definitively described.

The example presented in the written description training materials also indicates that an assay is known that will allow

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[1] It is noted that Applicants in the instant invention used the sequence of conserved region II to design and isolate the gene that encodes the instant alkaline α-amylase. Also see Attachment I, Nakajima et al., Comparison of amino acid sequences of eleven different α-amylases, Appl. Microbiol. Biotechnol. 23, 355-360, (1986).

one to identify the enzymes that have the stated activity. Likewise, the instant specification describes an assay that would allow one to identify enzymes that have the activity recited in the instant claimed invention. See page 17, lines 14-22 in the specification.

Every element that is present in the claim in the Example presented in the written description training materials has a comparable element in the instant claim. The underlying support in the disclosure for the claimed invention in the instant case is equal to or exceeds that support in the written description that is enumerated in the Example. Because the written description training materials expressly indicates that the example has adequate written description support, Applicants assert the present claim 3 is supported by adequate written description.

Further, the Court of Appeals for the Federal Circuit, in *University of California v. Eli Lilly*, 43 USPQ2d 1398, 1404 (Fed. Cir. 1997) citing *Fiers v. Revel*, 25 USPQ2d 1601 (Fed. Cir. 1993) held that adequate written description of a DNA "requires a precise definition, such as structure, formula, chemical name, or physical properties". The Board should consider that the Federal Circuit used alternative language in enumerating those things that constitute adequate written description. Thus, either a precise definition containing structural properties or a precise

definition containing physical properties is sufficient to show adequate written description.

Applicants contend that claim 3 and claims dependent from it are drawn to DNA having both defined structural elements and defined physical properties. The structural element of claim 3 derives from the amino acid sequence of SEQ ID NO: 2. Applicants have further provided an additional example of a species that fits into the claimed genus, i.e. SEQ ID NO: 2 with up to the 32 amino acids deleted from the N-terminus. (See page 12, lines 3-6).

Additional structural information can be gleaned from what was known in the art at the time of filing the application. It has long been held that the specification need not describe that which is known by one of skill in the art. See *In re Buchner*, 18 USPQ2d 1331 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.* 231 USPQ2d (Fed. Cir. 1986) and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 221 USPQ 481 (Fed. Cir. 1984). This knowledge of the skilled artisan supports written description.

Amylases are known to have four regions, designated regions I-IV that are highly conserved (see page 13, last line to page 14, line 3).[2] Thus, one of skill in the art would recognize that these conserved regions are portions of the sequence in which one would

[2] The Board should note that, in making the instant invention, Applicants used a primer designed from conserved region II that encodes the instant alkaline α-amylase. One of skill in the art can design primers based on conserved regions of proteins because these regions remain almost unchanged throughout "experiments of nature" conducted by evolution. Thus, these regions are thought important for enzyme function.

make only conserved amino acid substitutions (if any), and would not likely insert, delete or substitute one or more amino acids.

Claim 3 also lists the following physical properties;

1) an optimum activity at pH 8-9;

2) no change in enzymological properties from the enzyme having a sequence of SEQ ID NO:2;

3) the ability of this enzyme to hydrolyze 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6);

4) the enzyme does not hydrolyze pullulan.

Claims 4, 15, and 16, that are dependent from claim 3, have additional functional elements that further define subgenera.

Claim 4 also has a nucleotide sequence that regulates expression of a gene. Claim 15 is composed of a recombinant DNA containing the DNA of claim 3. Claim 16 is composed of a recombinant DNA containing the DNA of claim 4. Claim 20 has the further physical property limitation that the encoded protein has an isoelectric point higher than 8.5 when measured by isoelectric focusing electrophoresis.

Thus, according to *Fiers*, whose holding was cited with favor in *Lilly*, Applicants contend that they have met the written description requirement. In accordance with the rule set out in

Fiers, Applicants have provided precise definitions in claims 3, 4, 15, 16, and 20 i.e. the recitation of physical properties. Combined with the structural language which derives from SEQ ID NO: 2 and mutant proteins which have amino acids substituted, deleted or inserted provides structural language, Applicants have adequately defined the genus.

In *Lilly*, the Federal Circuit found that the University of California lacked written description because they had only disclosed cDNA for rat insulin in their written description, yet they generically claimed a recombinant plasmid containing cDNA for vertebrate insulin in their claims 1 and 2. The Court found that written description was lacking because the recitation of rat insulin was not sufficient to claim the entire genus of vertebrate insulin.

In *Lilly*, because the University of California had neither structural nor functional (physical properties) language in their claims, the Federal Circuit held that one of skill in the art would be unable to visualize or recognize what constituted the claimed genus.

The Federal Circuit held that the University of California could not have possession of a genus that could not be visualized or recognized. Further, the Federal Circuit found that the University of California had failed to adequately define the genes that fall within a genus of "mammalian insulin" or "vertebrate

insulin". Perhaps their question was, "what is the amino acid sequence of 'mammalian insulin' or 'vertebrate insulin'"? The University of California patent described a sequence for a rat insulin gene, but no sequence for any generic insulin, nor were any functional limitations described that indicated what was intended by "insulin".

On the other hand, one of skill in the art would be able to envision and recognize what constitutes the genus of claim 3. There is functional language as well as structural language in the claims, not mere naming of an enzyme as was done in *Lilly*. What is meant by "vertebrate insulin" is far less clear than what is meant by

> *"a DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9 and possessing an amino acid sequence which has been obtained by modifying an amino acid sequence described in SEQ ID NO:2 in a manner in which one or more amino acids are substituted, deleted, or inserted without changing enzymological properties of said amino acid sequence described in SEQ ID NO:2 and hydrolyzes 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) and does not hydrolyze pullulan".*

In *Lilly*, it is ambiguous whether "insulin" in the University of California's claims constituted a structural limitation on amino acid sequence or a functional limitation on activity. Furthermore, the scope of any possible functional limitation is open, as there is no specific level of activity recited. The

language of the claims in *Lilly* did not indicate that the insulin even had to be active. This is not the case with the instant claim 3.

The instant claim 3 recites cDNA that expresses alkaline α-amylase having the enzymological properties of a protein of SEQ ID NO: 2 and that has maximal activity at pH 8-9. The instant specification further describes how one would screen for enzymes that have maximal activities in the pH 8-9 range (see page 17, lines 14-22). This assay further describes quantitatively how one could measure the activity of these enzymes (one unit activity = amount of protein that produces a quantity of reducing sugar equivalent to 1 μmol of glucose). Further, Applicants have provided how one would screen recombinant microorganisms to identify those expressing an enzyme according to claim 3 (see page 9, lines 1-11).

When the structural features provided by SEQ ID NO: 2, the known conserved regions in amylases, the assays used to screen for mutant enzymes, and the limited number of amino acids (only 21 naturally encoded amino acids are known) are combined, one can only conclude that Applicants did have full possession of the full scope of their claims at the time of filing.

In conclusion, claims 3, 4, 15, 16 and 20 include both structural and functional language that definitively describes the genus encompassed by these appealed claims. The language of these

claims allows one of skill in the art to readily visualize and recognize the scope of the generic invention. Applicants have further provided methods for assaying how one tests enzymes that are variants of SEQ ID NO: 2 to easily ascertain species that fall into the claimed genus. Finally, Applicants have provided one species that falls into the claimed genus as well as described modification of those species that would fall into the claimed genus. Accordingly, Applicants have shown that they had possession of the full scope of claims 3, 4, 15, 16, and 20 at the time of filing the invention. It is respectfully requested that the honorable Board reverse the Examiner with respect to the written description rejection of these claims.

Issue 2

The specification, taken with what was known by one of skill in the art at the time of filing the present application, provides adequate written description of the following functional elements recited in claim 21:

(a) acts in a pH range of 5.0 to 11.0, with an optimum pH in the range of 8.0 to 9.0;

(b) is stable in a pH range of 5.0 to 10.5 and retains at least 50% of activity after treatment at 40°C for 30 minutes;

(c) acts in a temperature range of 20°C to 80°C, with an optimum temperature in the range of 45°C to 55°C;

(d) is stable at temperatures of 50°C or lower when treated for

30 minutes in a glycine-salt-sodium hydroxide buffer having pH 8.5;

(e) has a molecular weight of 50,000+5000 when measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis;

(f) has an isoelectric point of approximately 9.2 when measured by isoelectric focusing electrophoresis;

(g) is stable in the presence of K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Fe^{2+}, Fe^{3+}, or Al^{3+}; and

(h) is substantially free of inhibition by surfactants selected from the group consisting of sodium linear alkylbenzene sulfonates, sodium alkylsulfonate esters, sodium polyoxyethylene alkylsulfate esters, sodium alkylsulfonates, soaps and polyoxyethylene alkyl ethers.

Applicants assert that the description of the specification establishes that the inventors had possession of the full scope of claim 21 at the time of filing the application. All of the arguments presented above for Issue 1 apply to this Issue 2 as well. Claim 21 further recites structural and functional elements in addition to the structural and functional elements of claim 3. These elements include a pH range where the alkaline liquefying α-amylase has to be active, a temperature range where it is active, an isoelectric point, stability in the presence of metal ions and surfactants, and a molecular weight range. These elements are expressly described in the abstract of the PCT application from

which this application claims benefit. In particular, claim 21 recites a molecular weight range from 45,000 to 55,000 as an additional structural element (also disclosed in the abstract of the PCT application). This molecular weight range apprises one immediately of the collection of amino acids that are possible. In particular, the number of amino acids must be between 241 and 964 amino acids.[3] Every possible combination of amino acids that fits in this genus is immediately conceivable in this case. The additional functional limitations in claim 21 even further limit the genus. For example, polylysine of 241 to 964 amino acids would not have the requisite isoelectric point.

When the above-enumerated functional and physical property elements are combined with the written description of SEQ ID NO: 2, the conserved regions in amylases, the assays used to screen for mutant enzymes provided by the specification, and the limited number of amino acids (21 naturally encoded amino acids), one can only conclude that the claimed invention is well described by the specification. Further, it is clear that Applicants had full possession of the full scope of claim 21 at the time of filing of the instant application. It is respectfully requested that the honorable Board reverse the Examiner with respect to the written description rejection of claim 21.

[3] These numbers were obtained by taking 45,000g/mol/186.21g trp/mol trp (tryptophan is the heaviest amino acid) and 55000g/mol/57.05g

Issue 3

Claims 22-24, reciting a DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9, comprising at least one nucleotide sequence selected from a group of recited short sequences are not properly rejected under 35 USC §112, first paragraph for lacking description.

In *Fiers v. Revel*, 25 USPQ2d 1601 (Fed. Cir. 1993) the Federal Circuit held that adequate written description of a DNA "requires a precise definition, such as structure, formula, chemical name, or physical properties". As was previously explained, the Federal Circuit used alternative language in enumerating those things that constitute adequate written description. Thus, either a structural definition or a functional definition is sufficient to provide adequate written description. The present specification provides both of these in claims 22-24. Thus, claims 22-24 are adequately described in the specification.

Claims 22-24, which are directed to cloned DNA encoding an enzyme, have both structural and functional language. The structural element comes from sequences that comprise a primer or the reverse complement of the complementary strand primer. The alkaline α-amylase activity of the encoded enzyme and its maximal

activity at pH 8-9 of the enzyme encoded by the claimed DNA provides functional limitations.

The genus defined by claims 22-24 has a definitive structural element (i.e. short DNA sequences composed of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, or SEQ ID NO: 11). There is no ambiguity in this structural element. No variants in this structural element are claimed. The DNA that encodes the alkaline liquefying α-amylase, must have at least one of these enumerated sequences (or the reverse complement) present in them. The manner in which these short sequences relate to the entire structure of a DNA encoding an α-amylase of the invention is shown by use of primers of the recited sequences to isolate a DNA of the invention and in Figure 1. Thus, Applicants have defined a genus that can be immediately visualized or recognized. On this basis alone, Applicants have satisfied the Federal Circuit's requirement that one of skill in the art be able to visualize or recognize what constituted the claimed genus. See *University of California v. Eli Lilly*, 43 USPQ2d 1398 (Fed. Cir. 1997).

However, claims 22-24 contain additional functional features that further define the invention. The encoded protein must have α-amylase activity and this activity must be maximal at a pH from 8 to 9. What is meant by "alkaline liquefying activity" is defined in the specification at page 3 lines 5 *et seq.* of the

specification and an exemplary assay is provided at page 17, lines 14-22. The skilled artisan in enzymology well understands what is meant by a pH optimum of an enzyme activity.

In view of the above, the Board must conclude that the specification adequately describes the subject matter of claims 22-24 and that Applicants were in possession of an invention having the full scope of these claims. It is respectfully requested that the honorable Board reverse the Examiner with respect to this written description rejection.

Issue 4

The specification, taken with what was known by one of skill in the art at the time of filing the present application, enables the skilled artisan to make and use "an α-amylase having an amino acid sequence of SEQ ID NO:2 with one or more amino acids substituted, added, deleted or inserted" and having certain recited functional properties.

Applicants assert that claims 3, 4, 15, 16, and 20-21 reciting

> *a DNA encoding an α-amylase having an amino acid sequence of SEQ ID NO:2 with one or more amino acids substituted, added, deleted or inserted with maximal activity at a pH optimum of 8-9 and possessing an amino acid sequence which has been obtained by modifying an amino acid sequence described in SEQ ID NO:2 in a manner in which one or more amino acids are substituted, deleted, or inserted without changing enzymological properties of said amino acid sequence described in SEQ ID NO:2 and hydrolyzes 1,4-α-glucosidic*

> *linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) and does not hydrolyze pullulan*

are improperly rejected under 35 USC §112, first paragraph for not being fully enabled.

Regarding claims 3, 4, 15, 16, and 20-21, the Examiner recites in the Office Action of May 24, 2000:

> *The claims are broader than the enablement provided by the disclosure with regard to the huge number of all possible nucleic acid sequences encoding α-amylase having the specific desired characteristics.*

Applicants submit that the Examiner has failed to meet the burden of presenting a *prima facie* case as to why the claims would not be enabled. See *In re Wright*, 27 USPQ2d 1510 (Fed. Cir. 1993). *Wright*, citing *In re Marzocchi*, 169 USPQ 367, 369 *(CCPA 1971)* states

> *When rejecting a claim under the enablement requirement of section 112, the PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application; this includes, of course, providing sufficient reasons for doubting any assertions in the specification as to the scope of enablement. If the PTO meets this burden, the burden then shifts to the applicant to provide suitable proofs indicating that the specification is indeed enabling.*

The Examiner has failed to meet this initial burden. Even if the Examiner had met this burden, Applicants have provided an example that works. Absent some evidence from the Examiner that any

mutant enzyme would not work, one must assume that the full scope of the claimed invention is enabled by the specification. Consequently, claims 3, 4, 15, 16, and 20-21 are enabled for the full scope of the invention.

The Court of Appeals for the Federal Circuit in *In re Wands*, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) stated

> *Enablement is not precluded by the necessity for some experimentation such as routine screening. However, experimentation needed to practice the invention must not be undue experimentation. The key word is 'undue', not 'experimentation'.*

Applicants establish below that the amount of experimentation needed to practice the full-scope of the claimed invention is not 'undue'. Therefore, the present claims should be considered enabled by the present specification.

The Federal Circuit, in *Wands*, enumerated factors to be considered to ascertain whether or not claims are enabled. See *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988) at page 1404. These factors are:

1) the nature of the invention,

2) the breadth of the claims,

3) the quantity of experimentation needed to make or use the invention based on the disclosure of the invention

4) the amount of direction provided by the inventor,

5) the presence or absence of working examples,

6) the state of the prior art,

7) the relative skill of those in the art, and

8) the level of predictability in the art.

First, the nature of the invention is such that one of ordinary skill in the art would be able to make and use the invention commensurate in scope with the claims. The invention is cloned DNA encoding an α-amylase enzyme. Practice of the invention involves techniques such as the polymerase chain reaction (PCR), site directed mutagenesis, gene-splicing, and other associated techniques to generate mutant enzymes. Recombinant DNA manipulation techniques, such as site-directed mutagenesis and PCR are well known and routine in the art. In fact, kits for performing these techniques are commercially available. These kits allow one to practice the invention easily. Further, the disclosure has provided guidance as to how one would go about practicing the invention, including how one would screen for microorganisms to identify those harboring DNA of the invention (see page 9, lines 1-11) and how one would test for activity of the encoded protein (see page 17, lines 14-22).

Second, the breadth of claims 3, 4, 15, 16, 20, and 21 is large, encompassing many variations of SEQ ID NO: 2. However, the breadth of these claims is considerably constrained by the functional limitations recited in claim 3 as the particulars of the activity of the encoded enzyme. An important limitation is

that the enzymological properties of the enzyme must be the same as those of a protein having the amino acid sequence of SEQ ID NO: 2. One of skill in the art can readily determine, by the assay described at page 17, lines 14-22, whether any variant of SEQ ID NO: 2 is the same as the parental enzyme in this regard and thus the breadth of claim 3 is considerably reduced. The breadth of claim 3 is further reduced by a limitation that the maximum activity be observed at pH 8-9, that certain recited substrates are hydrolyzable by the enzymes produced, and that one substrate is not hydrolyzable.

Third, the state of the prior art also would allow one to make and use the invention commensurate with the claimed invention. α-amylases are well known in the prior art. There are known to be four conserved regions (designated regions I-IV) in α-amylases. One of skill in the art would recognize that these are not regions where one would be likely to make additions, substitutions (other than perhaps conservative substitutions) and deletions. The inventors of the instant invention have further used one of these conserved regions (region II) to design a primer so that the gene encoding the alkaline liquefying α-amylase could be isolated from various species of microorganisms. Thus, one of skill in the art would recognize that knowledge in the prior art is sufficiently high that the skilled artisan would be able to

practice the invention commensurate in scope with the claimed invention.

Fourth, it is generally acknowledged that the level of skill in the biotechnology art is high. Usually, the practicing artisan possesses a Ph.D. The Patent Office itself calls the recombinant art "a complex technology". Accordingly, the skill of those who practice in this art must necessarily be advanced in order to practice this "complex technology".

Fifth, the next *Wands* factor is predictability in the art. Applicants admit one can not immediately tell from the primary amino acid sequence whether or not a given amino acid sequence for a liquefying α-amylase would be active at the same level as an enzyme having the amino acid of SEQ ID NO:2. Thus, the predictability of function from primary structure is low.

However, obtaining an active and operable embodiment of the invention can easily be achieved by screening a library of mutants by the method indicated on page 17, lines 14-22 of the instant specification. The holding in *Wands* expressly stated that such screening was not "undue" experimentation. Screening is expected in the molecular biology art. Applicants, in this particular instance, have devised a spectrophotometric means of testing any possible mutant that allows for high throughput screening of mutants (see page 17, lines 14-22). Use of spectrophotometric assays for screening is routine in the art.

Further, recombinant techniques such as site-directed mutagenesis, the polymerase chain reaction and other recombinant techniques, and sequencing are well known and quite predictable. Kits are common in the art that allow one to practice these techniques and obtain consistent and positive results time after time. Further, Applicants have provided disclosure how one would select the mutants (see page 9, lines 1-11). As to the limitation on isoelectric point (pI) in claim 21, a theoretical pI is very simple to calculate from an amino acid sequence. Computer programs were available at the time filing the application that allowed one to calculate pIs from an amino acid sequence. See attachment II Skoog et al., "Calculation of the Isoelectric Points of Polypeptides From the Amino Acid Composition", *Computer Corner*, 5(4), (1986). Thus, even though predictability may be low based on the primary amino acid sequence, the screening techniques that are used to discover active enzyme variants are trivial. Applicants submit that it is highly likely that at least one active variant enzyme would be isolated in any single experiment of this type. Thus, predictability of success in a "mutation-screening" experiment is high.

Sixth, Applicants have provided sufficient direction so that one of skill in the art could practice the invention. In particular, Applicants have provided assays as to how mutant microorganisms can be selected and also how the mutant enzymes can

be assayed to ascertain their activity (see page 9, lines 1-11 and see page 17, lines 14-22, respectively). Recombinant techniques, such as site-directed mutagenesis are well known in the art and thus it would have been redundant for Applicants to have detailed these techniques in detail in the written description. See *In re Buchner*, 18 USPQ2d 1331 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.* 231 USPQ2d (Fed. Cir. 1986) and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 221 USPQ 481 (Fed. Cir. 1984).

Further, because the level of skill is high in the recombinant art, and because the amount of guidance or direction needed to enable the invention is inversely related to the amount of knowledge in the state of the art, a lot of guidance is not necessary (see *In re Fischer*, 166 USPQ 18, 24 (CCPA 1970)). Accordingly, Applicants have provided sufficient guidance so that one of skill in the art would be able to practice the full scope of the claimed invention.

Seventh, Applicants have provided one working example of an enzyme that cleaves certain carbohydrate bonds recited in the claims and operates maximally at pH 8-9. A screening method that is simple to perform is demonstrated. Recombinant techniques to generate variant enzymes starting from the cloned DNA obtained in the working example are well known. One of skill in the art would recognize that great changes should not be made in parts of the

sequence that are highly conserved (such as regions I-IV). Further, the claims have constraints on them that would direct one to the sequences that fall into the claimed genus, such as the activity being at a maximum from 8-9, the enzyme retaining its enzymatic activity, and the enzyme cleaving 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) and does not hydrolyze pullulan. In view of the above, the one working example provided should be sufficient to justify a generic claim encompassing the disclosed sequence and similar analogues.

Eighth, the quantity of experimentation needed to make and use the invention based on the disclosure is not overly large. Following the working examples of the specification, the prototype DNA of SEQ ID NO: 2 can be obtained from a deposited *Bacillus* strain in a few days time. Mutation and screening experiments as expected in the art can typically be performed within an additional week or two. A skilled artisan in molecular biology does not consider this a large amount of experimentation. The predictability of recombinant DNA manipulation and screening techniques used to practice the invention is also high, and importantly such experiments are expected to be performed by the skilled artisan. The guidance provided by the disclosure is

sufficient so that one of skill in the art could practice the invention without designing any new assay. Applicants have described how one would select for mutant microorganisms and Applicants have provided how one would assay any mutant enzyme to test for activity, a constraint on the breadth of the claims (see page 9, lines 1-11 and see page 17, lines 14-22, respectively). The prior art provides guidance as to structural portions of the enzyme that are important to conserve. In view of the above, the experimentation that would be necessary to practice the invention commensurate in scope with the claims would not be undue. Thus, the claimed invention is fully enabled throughout the full scope of the claims. It is respectfully requested that the honorable Board reverse the Examiner with respect to the enablement rejection of claims 3, 4, 15, 16 and 20-21.

Issue 5

Claims 22-24 claiming DNA molecules encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9, comprising at least one nucleotide sequence selected from the group consisting of the primers disclosed in the invention or the reverse complement of primers disclosed from the complementary strand are improperly rejected under 35 USC §112, first paragraph for lack of enablement.

The Examiner in the Office Action of December 4, 2000 recites

> *. . . the specification . . . does not reasonably provide enablement for a DNA comprising a fragment of about 20 base pairs*

Applicants submit that the Examiner has failed to meet the burden of presenting a *prima facie* case as to why the claims would not be enabled. See *In re Wright*, 27 USPQ2d 1510 (Fed. Cir. 1993). *Wright*, citing *In re Marzocchi*, 169 USPQ 367, 369 *(CCPA 1971)* states

> *When rejecting a claim under the enablement requirement of section 112, the PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application; this includes, of course, providing sufficient reasons for doubting any assertions in the specification as to the scope of enablement. If the PTO meets this burden. the burden then shifts to the applicant to provide suitable proofs indicating that the specification is indeed enabling.*

The Examiner has failed to meet this initial burden. Even if the Examiner had met this burden, Applicants have provided an example that works. Absent some evidence from the Examiner that any mutant enzyme would not work, one must assume that the full scope of the claimed invention is enabled by the specification. Consequently, claims 22-24 are enabled for the full scope of the invention.

Furthermore, consideration of the *Wands* factors will show that undue experimentation is not required to practice the invention set forth in claims 22-24. The nature of the invention,

the level of skill in the art, the predictability of the art, the state of the prior art and the amount of experimentation to practice the invention are all the same as for claims 3, 4, 15, 16, and 20-21. Thus the arguments on these points presented as to Issue 4 apply here as well.

The breadth of claims 22-24 differs from that of the other claims on appeal. Claims 22-24 recite specific structural features, i.e. inclusion of certain short nucleotide sequences that are not recited in the other claims. The Board is reminded that the DNA claimed in claims 22-24 comprises these sequences. One of skill in the art recognizes that it is not likely that a DNA including only these sequences would encode an enzyme possessing α-amylase activity having an optimum level at pH 8-9. Rather, many more amino acids would be needed to complete the enzyme structure.

The disclosure of the specification describes this. In particular, the working examples show that the short DNA sequences recited in the claims are used in a PCR reaction upon a template DNA from some bacterium that expresses a relevant enzyme. The product is the DNA of the invention. (See, Examples 4-6).

Bacteria expressing the relevant enzyme can be identified by Southern Blotting with DNA of "Fragment A", described in Figure 1 (See p. 14, line 12 and Example 3). Species of bacteria that are also possible candidates for use in this process are described on

page 2 of the specification. As described above, the assay for activity described in the application allows the skilled artisan to easily distinguish a DNA that encodes an enzyme having alpha-amylase activity, with optimum activity at pH 8-9, from embodiments outside the scope of the claims.

In view of the above, the invention of claims 22-24 should be considered well enabled by the instant specification. Accordingly, the rejection of claims 22-24 under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement, should be reversed. Thus, it is respectfully requested that the honorable Board reverse the Examiner with respect to this enablement rejection.

Issue 6

Claims 3, 4, 15, 16, and 20-24 are patentable over Ara '367 in view of Tsukamoto et al. or Yuuki et al.

Disclosure of Ara '367

Ara '367 discloses a liquefying alkaline α-amylase that is isolated from *Bacillus sp.* KSM-AP1378. Ara '367 discloses the 10 terminal amino acids from the N-terminal sequence of the alkaline α-amylase (via Edman degradation). Ara '367 does not disclose or suggest the nucleotide or complete amino acid sequence of a liquefying alkaline α-amylase.

Disclosure of Tsukamoto et al.

Tsukamoto et al. disclose the nucleotide sequence of the maltohexose-producing α-amylase gene from *Bacillus sp.* #707 Tsukamoto et al. also disclose a comparison of the nucleotide and amino acid sequences of their nucleotide sequence to other bacterial liquefying α-amylase sequences. Tsukamoto et al. does not disclose or suggest a liquefying alkaline α-amylase.

Disclosure of Yuuki et al.

Yuuki et al. disclose the complete nucleotide sequence for heat-stable and pH-stable α-amylase of *Bacillus lichenformis*. Yuuki et al. also disclose a comparison of the amino acid sequences of three bacterial liquefying a-amylases deduced from the DNA sequences. Yuuki et al. does not disclose or suggest a liquefying alkaline α-amylase having a pH optimum of activity from pH 8 to 9.

Removal of Ara '367 in view of Tsukamoto et al. or Yuuki et al

The Examiner has failed to make out a *prima facie* case of obviousness with regard to the 35 USC §103(a) rejection over Ara '367 in view of Tsukamoto et al. or Yuuki et al. Three criteria must be met to make out a *prima facie* case of obviousness.

1) There must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings.

2) There must be a reasonable expectation of success.

3) The prior art reference (or references when combined) must teach or suggest all the claim limitations.

See MPEP §2142 and *In re Vaeck*, 20 USPQ2d 1438 (CAFC, 1991). In particular, the Examiner has failed to meet the third element to make a *prima facie* obviousness rejection. The prior art references do not teach or suggest all of the claim elements. Claim 3 will be used to illustrate this. Claim 3 claims

> *3. (Twice Amended) An isolated DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9 and possessing an amino acid sequence obtained by modifying an amino acid sequence described in SEQ ID NO: 2 in a manner in which one or more amino acids are substituted, deleted, or inserted without changing the enzymological properties of the protein having said amino acid sequence described in SEQ ID NO:2 and the protein hydrolyzes 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) and does not hydrolyze pullulan.*

This is the broadest of the appealed claims. Claim 3 claims an amino acid sequence (SEQ ID NO: 2) or a sequence that has been modified from that amino acid sequence without changing enzymological properties of the wild-type enzyme. None of the

references disclose or suggest a complete amino acid sequence that has the properties of the instant invention (*i.e.*, operates at a pH optimum of 8-9 and hydrolyzes 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) and does not hydrolyze pullulan). As no single reference discloses these properties, no combination of these references can disclose or suggest them, either. Accordingly, a *prima facie* case of obviousness has not been made. The rejection is inapposite.

Moreover, claims 20 and 21 have additional features that are neither disclosed nor suggested by the above cited references. Claim 20 claims "an isolectric point higher than 8.5 when measured by isoelectric focusing electrophoresis". The references mention nothing of this feature. Finally, claim 21 has a series of features that are neither disclosed nor suggested by the cited references. Among these features are a pH optimum in the range of 8.0 to 9.0; stability in a pH range of 5.0 to 10.5 with a retention of at least 50% of activity after treatment at 40°C for 30 minutes; the ability to act in a temperature range of 20°C to 80°C, with an optimum temperature in the range of 45°C to 55°C; stability at temperatures of 50°C or lower when treated for 30 minutes in a glycine-salt-sodium hydroxide buffer having pH 8.5; with a molecular weight of 50,000±5000 when measured by sodium

dodecyl sulfate polyacrylamide gel electrophoresis; an isoelectric point of approximately 9.2 when measured by isoelectric focusing electrophoresis; stability in the presence of K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Fe^{2+}, Fe^{3+}, or Al^{3+}; and the enzyme is substantially free of inhibition by surfactants selected from the group consisting of sodium linear alkylbenzene sulfonates, sodium alkylsulfonate esters, sodium polyoxyethylene alkylsulfate esters, sodium alkylsulfonates, soaps and polyoxyethylene alkyl ethers. Accordingly, these additional features render the rejection inapposite. Reversal of the rejection is appropriate and respectfully requested.

IX. Conclusion

For the reasons advanced above, it is respectfully submitted that all claims in this application are allowable. Thus, favorable reconsideration and reversal of the Examiner's rejection of claims 3, 4, 15, 16, and 20-24 under 35 U.S.C. § 112, first paragraph, for alleged lack of written description and for alleged lack of enablement, by the Honorable Board of Patent Appeals and Interferences, is respectfully solicited.

Pursuant to 37 C.F.R. §§ 1.17 and 1.136(a), Applicant(s) respectfully petition(s) for a three (3) month extension of time for filing a reply in connection with the present application, and the required fee of $920.00 is attached hereto.

The required Appeal Brief fee in the amount of $320.00 and a three-month extension fee in the amount of $920.00 are attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

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0014-0188P

Attachment: APPENDIX
Attachment

X. Appendix (Appealed claims)

3. (Twice Amended) An isolated DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9 and possessing an amino acid sequence obtained by modifying an amino acid sequence described in SEQ ID NO: 2 in a manner in which one or more amino acids are substituted, deleted, or inserted without changing the enzymological properties of the protein having said amino acid sequence described in SEQ ID NO:2 and the protein hydrolyzes 1,4-α-glucosidic linkages in starches, amylose, amylopectin, and degradation products thereof and in amylose forms: glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5) and maltohexose (G6) and does not hydrolyze pullulan.

4. (Twice Amended) The DNA molecule of claim 2 or 3^4, further comprising a nucleotide sequence for regulating expression of said isolated DNA molecule.

15. (Amended) A recombinant DNA molecule comprising the isolated DNA molecule of claim 3.

16. (Amended) A recombinant DNA molecule comprising the

isolated DNA molecule of claim 4.

20. (Amended) The DNA molecule of claim 3, wherein said encoded protein has an isolectric point higher than 8.5 when measured by isoelectric focusing electrophoresis.

21. The DNA molecule of claim 3, wherein said encoded protein:

acts in a pH range of 5.0 to 11.0, with an optimum pH in the range of 8.0 to 9.0;

is stable in a pH range of 5.0 to 10.5 and retains at least 50% of activity after treatment at 40°C for 30 minutes;

acts in a temperature range of 20°C to 80°C, with an optimum temperature in the range of 45°C to 55°C;

is stable at temperatures of 50°C or lower when treated for 30 minutes in a glycine-salt-sodium hydroxide buffer having pH 8.5;

has a molecular weight of 50,000±5000 when measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis;

has an isoelectric point of approximately 9.2 when measured by isoelectric focusing electrophoresis;

is stable in the presence of K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Fe^{2+}, Fe^{3+}, or Al^{3+}; and

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[1] Note that although this claim depends from claims 2 and 3, only claim 3 is being appealed.

is substantially free of inhibition by surfactants selected from the group consisting of sodium linear alkylbenzene sulfonates, sodium alkylsulfonate esters, sodium polyoxyethylene alkylsulfate esters, sodium alkylsulfonates, soaps and polyoxyethylene alkyl ethers.

22. (Amended) An isolated DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9 comprising at least one nucleotide sequence that is selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 7, SEQ ID NO: 3, SEQ ID NO: 6 and SEQ ID NO: 9.

23. (Amended) An isolated DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9 comprising at least one nucleotide sequence that is the reverse complement of a sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 4 and SEQ ID NO: 11.

24. (Amended) An isolated DNA molecule encoding a protein exhibiting alkaline liquefying α-amylase activity at a pH optimum of 8-9 comprising at least one nucleotide sequence selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 7, SEQ ID NO: 3, SEQ ID NO: 6 and SEQ ID NO: 9, and also comprising at least one nucleotide sequence that is the reverse complement of a sequence

selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 4 and SEQ ID NO: 11.

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Computer Corner

(ATTACHMENT II)

Calculation of the isoelectric points of polypeptides from the amino acid composition

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The isoelectric point (p*I*) of a polypeptide is of practical importance in many separation procedures, both analytical and preparative. The p*I* is defined as the pH where the net charge of the peptide is zero. Contributing to the net charge are terminal amino acids and charged amino acid side-chains within the sequence. Therefore, by using the primary structure, a theoretical p*I* value can be calculated. In this Corner a Pascal program is presented for the calculation of the p*I* value of a known carbohydrate-free polypeptide.

The fundamental Henderson–Hasselbach relationship:

$$pH = pK_a' + \log (\text{basic species/acidic species})$$

describes the ratio of conjugate acid and base in a solution. Using this equation an ionization ratio at a specific pH can be calculated from the pK_a value.

The contribution, C_{pos}, for a positively charged amino acid residue (arg, his or lys) or the N-terminal amino acid at a certain pH (pH_{test}) is thus calculated by

$$C_{pos} = 1/(1 + \alpha)$$

where $\alpha = 10^{(pH_{test} - pK_a')}$

In the case of a negatively charged amino acid (asp, cys, glu or tyr) or the C-terminal amino acid the contribution, C_{neg}, is calculated by

$$C_{neg} = \alpha/(1 + \alpha)$$

The pK_a' values from amino acid side chains or for the amino/carboxyl end can be found in literature[1]. A net charge for every pH_{test} can thus be calculated from the number of positive and negative amino acids in the polypeptide. Using Newton–Raphson's iterative procedure it is possible to calculate the pH where the net charge is zero (p*I*). The iteration starts with two pH_{test} values (the secant method) and is repeated, while the successive approximations of p*I* converge with increasing accuracy.

The number of charged amino acids, number of chains and their terminal amino acids are input values from keyboard. Starting with the two extreme pH_{test} values 1 and 14 a p*I* usually is generated within 20 iterations (accuracy 0.0005).

TABLE I. Comparison of p*I* values generated by the Pascal program and literature values.

Protein	p*I*	
	Calculated	Literature
Human growth hormone (hGH)	5.3	5.2[2,3],5.5[4]
Asp 152 desamido hGH	5.1	5.1[3]
Glu 137, asp 152 desamido hGH	5.0	4.9[3]
20K hGH	5.6	5.4[2],5.9[5]
24K hGH (1–134 + 141–191) (plasmin digested hGH)	5.1	< p*I* for hGH[6]
Desialylated human antithrombin III*	5.3-6.0	5.5-5.8[7]
Thrombin, bovine	6.7	5.6-7.1[8] **

* p*I* interval calculated after assuming different degree of desamidation within the antithrombin III molecule.
** Values for human thrombin.

Derived p*I* values are in many cases comparable to reported values (see Table I). Discrepancies will arise due to, for instance, carbohydrate moieties or modifications of amino acid residues.

The program is written in Apple LisaPascal but will work directly on any computer with an UCSD-Pascal compiler and with only minor changes on any Pascal environment. Program code length is 3.6 kB. With only minor modifications the program can also be used to generate theoretical titration curves.

0165-9936/86/$02.00.

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B. Skoog and A. Wichman are at KabiVitrum AB, Research Department, S-112 87 Stockholm, Sweden.

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{ ============================================================ }
{ Isoelectric point determination of polypeptides              }
{ ============================================================ }

PROGRAM Iso_point;

CONST   pKa3_arg=12.5; pKa3_asp=3.9; pKa3_cys=8.3;
        pKa3_glu=4.3;  pKa3_his=6.0; pKa3_lys=10.5;
        pKa3_tyr=10.1;
        Max_chains=5;
        Max_iterations=100;
        pH1=1;      pH2=14;        Accuracy=0.00005;

        All = 'alaargasnaspcysglngluglyhisileleulysmetpheproserthrtrptyrva

TYPE    Str3    =       String[3];
        Str80   =       String[80];
        Aminoacid   =   ARRAY [1..Max_chains] OF REAL;
        Turn        =   ARRAY [1..Max_iterations] OF REAL;
        N_values    =   ARRAY [1..20] OF REAL;
        C_values    =   ARRAY [1..20] OF REAL;


VAR     Name        :   Str80;
        Test        :   Str3;

        Num_arg, Num_asp, Num_cys,
        Num_glu, Num_his, Num_lys,
        Num_tyr, Num_chains     : INTEGER;

        N_term,
        C_term  : Aminoacid;

        Test_pH,
        Charge  : Turn;

        TermN,TermC         :   REAL;
        Number, N           :   INTEGER;
        NtermpK             :   N_values;
        CtermpK             :   C_values;
        Sum,Temp            :   REAL;



FUNCTION        Neg_charge (pK_value    : REAL;
                            pH          : Turn)       : REAL;
BEGIN
        Neg_charge:=(exp(2.3026*((pH[N])-(pK_value))))/
                    (1+(exp(2.3026*((pH[N])-(pK_value)))));
END;    (Neg_charge)


FUNCTION        Pos_charge (pK_value    : REAL;
                            pH          : Turn)       : REAL;
BEGIN
        Pos_charge:=(1/(exp(2.3026*(((pH[N])-(pK_value))))+1));

END;    (Pos_charge)


PROCEDURE Findend;

BEGIN
         NtermpK[1]:=9.9;NtermpK[2]:=9.0;NtermpK[3]:=8.8;NtermpK[4]:=9.8;
         NtermpK[5]:=10.8;NtermpK[6]:=9.1;NtermpK[7]:=9.7;NtermpK[8]:=9.9;
         NtermpK[9]:=9.2;NtermpK[10]:=9.8;NtermpK[11]:=9.7;NtermpK[12]:=9.0;
         NtermpK[13]:=9.3;NtermpK[14]:=9.2;NtermpK[15]:=10.6;NtermpK[16]:=9.2;
         NtermpK[17]:=9.1;NtermpK[18]:=9.4;NtermpK[19]:=9.1;NtermpK[20]:=9.7;
         CtermpK[1]:=2.4;CtermpK[2]:=2.2;CtermpK[3]:=2.1;CtermpK[4]:=2.1;
         CtermpK[5]:=1.7;CtermpK[6]:=2.2;CtermpK[7]:=2.2;CtermpK[8]:=2.4;
         CtermpK[9]:=1.8;CtermpK[10]:=2.3;CtermpK[11]:=2.3;CtermpK[12]:=2.2;
         CtermpK[13]:=2.1;CtermpK[14]:=2.2;CtermpK[15]:=2.0;CtermpK[16]:=2.2;
         CtermpK[17]:=2.1;CtermpK[18]:=2.4;CtermpK[19]:=2.2;CtermpK[20]:=2.3;
         Number:=Pos(Test,All);
         Number:=(Number+2) DIV 3;
         TermN:=NtermpK[Number];
         TermC:=CtermpK[Number];
END; (Findend)


PROCEDURE Check(VAR  Instr : Str3);

VAR     Count  :        INTEGER;

BEGIN
         FOR count:= 1 TO 3 DO IF Instr[count] IN ['A'..'Z'] THEN
           Instr[count] := CHR(ORD(Instr[count])+32);
END;    (Check)


PROCEDURE Input;

VAR     n       :       1..Max_chains;

BEGIN
        Write('Name of protein/peptide: ');
        Readln(Name);
        Writeln;
        Writeln('Please, enter the number of charged amino acids');
        Write('arg: ');     Readln(Num_arg);
        Write('asp: ');     Readln(Num_asp);
        Write('cys: ');     Readln(Num_cys);
        Write('glu: ');     Readln(Num_glu);
        Write('his: ');     Readln(Num_his);
        Write('lys: ');     Readln(Num_lys);
        Write('tyr: ');     Readln(Num_tyr);
        Writeln;
        Write('Number of polypeptide chains: ');
        Readln(Num_chains);
        FOR n:=1 TO Num_chains DO
        BEGIN
            Write('Chain',n:2,' N-terminal amino acid: ');
            Readln(Test);
            Check(Test);
            Findend;
            N_term[n]:=TermN;
            Write('Chain',n:2,' C-terminal amino acid: ');
            Readln(Test);
            Check(Test);
            Findend;
            C_term[n]:=TermC;
        END;
END;    (Input)


PROCEDURE       Find_charge;

VAR     i       :       1..Max_chains;

BEGIN
        Sum:=0;
        Sum:=Sum-(Num_asp)*(Neg_charge(pKa3_asp,Test_pH));
        Sum:=Sum-(Num_glu)*(Neg_charge(pKa3_glu,Test_pH));
        Sum:=Sum-(Num_cys)*(Neg_charge(pKa3_cys,Test_pH));
        Sum:=Sum-(Num_tyr)*(Neg_charge(pKa3_tyr,Test_pH));
        Sum:=Sum+(Num_his)*(Pos_charge(pKa3_his,Test_pH));
        Sum:=Sum+(Num_lys)*(Pos_charge(pKa3_lys,Test_pH));
        Sum:=Sum+(Num_arg)*(Pos_charge(pKa3_arg,Test_pH));
        FOR i:=1 to Num_chains DO
        BEGIN
                Temp:=(Pos_charge(N_term[i],Test_pH));
                Sum:=Sum+Temp;
                Temp:=(Neg_charge(C_term[i],Test_pH));
                Sum:=Sum-Temp;
                Temp:=Sum;
        END;
        Charge[N]:=Temp;
END;    (Find_charge)


PROCEDURE       Iterate;

VAR     Factor, Diff    :       REAL;

BEGIN
        N:=1;   Test_pH[N]:=pH1;        Find_charge;
        N:=2;   Test_pH[N]:=pH2;        Find_charge;
        REPEAT
            N:=N+1;
            BEGIN
               Factor:=(Test_pH[N-1])-(Test_pH[N-2]))/((Charge[N-1])-(Charge[N-2]));
               Test_pH[N]:=(Test_pH[N-1])-(Charge[N-1]*Factor);
               Find_charge;
               Temp:=(Charge[N])-(Charge[N-1]);
               Diff:=ABS(Temp);
            END;
        UNTIL (Diff)<=(Accuracy);
        Writeln;
        Writeln('Isoelectric point calculated to pH= ',Test_pH[N]:5:3);
        Writeln;
        Writeln('Accuracy= ',Accuracy:7:5,' pH units');
        Writeln('Number of iterations= ',N);
END;    (Iterate)


BEGIN   (main)
        Input;
        Iterate;
END.
```

```
{ ================================================================ }
{ Isoelectric point determination of polypeptides                  }
{ ================================================================ }

PROGRAM Iso_point;

CONST   pKa3_arg=12.5; pKa3_asp=3.9; pKa3_cys=8.3;
        pKa3_glu=4.3;  pKa3_his=6.0; pKa3_lys=10.5;
        pKa3_tyr=10.1;
        Max_chains=5;
        Max_iterations=100;
        pH1=1;      pH2=14;        Accuracy=0.00005;

        All = 'alaargasnaspcysglnglugly hisileleulysmetpheproserthrtrptyrva

TYPE    Str3     =      String[3];
        Str80    =      String[80];
        Aminoacid   =   ARRAY [1..Max_chains] OF REAL;
        Turn        =   ARRAY [1..Max_iterations] OF REAL;
        N_values    =   ARRAY [1..20] OF REAL;
        C_values    =   ARRAY [1..20] OF REAL;


VAR     Name        :   Str80;
        Test        :   Str3;

        Num_arg, Num_asp, Num_cys,
        Num_glu, Num_his, Num_lys,
        Num_tyr, Num_chains     : INTEGER;

        N_term,
        C_term  : Aminoacid;

        Test_pH,
        Charge  : Turn;

        TermN,TermC         :   REAL;
        Number, N           :   INTEGER;
        NtermpK             :   N_values;
        CtermpK             :   C_values;
        Sum,Temp            :   REAL;



FUNCTION      Neg_charge (pK_value     : REAL;
                          pH           : Turn)       : REAL;

BEGIN
        Neg_charge:=(exp(2.3026*((pH[N])-(pK_value))))/
                    (1+(exp(2.3026*((pH[N])-(pK_value)))));
END;    {Neg_charge}


FUNCTION      Pos_charge (pK_value     : REAL;
                          pH           : Turn)       : REAL;
BEGIN
        Pos_charge:=(1/(exp(2.3026*(((pH[N])-(pK_value))))+1));

END;    {Pos_charge}
```

```
        Charge  : Turn;

        TermN,TermC         :   REAL;                                   PROCEDU
        Number, N           :   INTEGER;
        NtermpK             :   N_values;                               VAR
        CtermpK             :   C_values;
        Sum,Temp            :   REAL;                                   BEGIN


FUNCTION        Neg_charge (pK_value    : REAL;
                            pH          : Turn)       : REAL;

BEGIN
        Neg_charge:=(exp(2.3026*((pH[N])-(pK_value))))/
                    (1+(exp(2.3026*((pH[N])-(pK_value)))));
END;    (Neg_charge)


FUNCTION        Pos_charge (pK_value    : REAL;
                            pH          : Turn)       : REAL;
BEGIN
        Pos_charge:=(1/(exp(2.3026*(((pH[N])-(pK_value))))+1));         END;

END;    (Pos_charge)

                                                                        PROCEDU
PROCEDURE Findend;                                                      VAR

BEGIN                                                                   BEGIN
          NtermpK[1]:=9.9;NtermpK[2]:=9.0;NtermpK[3]:=8.8;NtermpK[4]:=9.8;
          NtermpK[5]:=10.8;NtermpK[6]:=9.1;NtermpK[7]:=9.7;NtermpK[8]:=9.8;
          NtermpK[9]:=9.2;NtermpK[10]:=9.8;NtermpK[11]:=9.7;NtermpK[12]:=9.0;
          NtermpK[13]:=9.3;NtermpK[14]:=9.2;NtermpK[15]:=10.6;NtermpK[16]:=9.2;
          NtermpK[17]:=9.1;NtermpK[18]:=9.4;NtermpK[19]:=9.1;NtermpK[20]:=9.7;
          CtermpK[1]:=2.4;CtermpK[2]:=2.2;CtermpK[3]:=2.1;CtermpK[4]:=2.1;
          CtermpK[5]:=1.7;CtermpK[6]:=2.2;CtermpK[7]:=2.2;CtermpK[8]:=2.4;
          CtermpK[9]:=1.8;CtermpK[10]:=2.3;CtermpK[11]:=2.3;CtermpK[12]:=2.2;
          CtermpK[13]:=2.1;CtermpK[14]:=2.2;CtermpK[15]:=2.0;CtermpK[16]:=2.2;
          CtermpK[17]:=2.1;CtermpK[18]:=2.4;CtermpK[19]:=2.2;CtermpK[20]:=2.3;
          Number:=Pos(Test,All);
          Number:=(Number+2) DIV 3;
          TermN:=NtermpK[Number];
          TermC:=CtermpK[Number];
END; (Findend)


                                                                        END;
PROCEDURE Check(VAR  Instr : Str3);

VAR     Count   :       INTEGER;
                                                                        BEGIN
BEGIN
          FOR count:= 1 TO 3 DO IF Instr[count] IN ['A'..'Z'] THEN
            Instr[count] := CHR(ORD(Instr[count])+32);                  END.
END;    (Check)
```

```
:=======  }
         }
:=======  }

PROCEDURE Input;

VAR      n       :     , 1..Max_chains;

BEGIN
         Write('Name of protein/peptide: ');
         Readln(Name);
         Writeln;
         Writeln('Please, enter the number of charged amino acids');
         Write('arg: ');      Readln(Num_arg);
         Write('asp: ');      Readln(Num_asp);
         Write('cys: ');      Readln(Num_cys);
         Write('glu: ');      Readln(Num_glu);
         Write('his: ');      Readln(Num_his);
         Write('lys: ');      Readln(Num_lys);
         Write('tyr: ');      Readln(Num_tyr);
         Writeln;
         Write('Number of polypeptide chains: ');
         Readln(Num_chains);
         FOR n:=1 TO Num_chains DO
         BEGIN
             Write('Chain',n:2,' N-terminal amino acid: ');
             Readln(Test);
             Check(Test);
             Findend;
             N_term[n]:=TermN;
             Write('Chain',n:2,' C-terminal amino acid: ');
             Readln(Test);
             Check(Test);
             Findend;
             C_term[n]:=TermC;
         END;
END;     (Input)


PROCEDURE        Find_charge;

VAR      i       :        1..Max_chains;

BEGIN
         Sum:=0;
         Sum:=Sum-(Num_asp)*(Neg_charge(pKa3_asp,Test_pH));
         Sum:=Sum-(Num_glu)*(Neg_charge(pKa3_glu,Test_pH));
         Sum:=Sum-(Num_cys)*(Neg_charge(pKa3_cys,Test_pH));
         Sum:=Sum-(Num_tyr)*(Neg_charge(pKa3_tyr,Test_pH));
         Sum:=Sum+(Num_his)*(Pos_charge(pKa3_his,Test_pH));
         Sum:=Sum+(Num_lys)*(Pos_charge(pKa3_lys,Test_pH));
         Sum:=Sum+(Num_arg)*(Pos_charge(pKa3_arg,Test_pH));
         FOR i:=1 to Num_chains DO
         BEGIN
                 Temp:=(Pos_charge(N_term[i],Test_pH));
                 Sum:=Sum+Temp;
                 Temp:=(Neg_charge(C_term[i],Test_pH));
                 Sum:=Sum-Temp;
                 Temp:=Sum;
         END;
         Charge[N]:=Temp;
END;     (Find_charge)


PROCEDURE        Iterate;
```

```
            C_term[n]:=[redacted]mC;
        END;
END;    {Input}


PROCEDURE       Find_charge;

VAR     i       :       1..Max_chains;

BEGIN
        Sum:=0;
        Sum:=Sum-(Num_asp)*(Neg_charge(pKa3_asp,Test_pH));
        Sum:=Sum-(Num_glu)*(Neg_charge(pKa3_glu,Test_pH));
        Sum:=Sum-(Num_cys)*(Neg_charge(pKa3_cys,Test_pH));
        Sum:=Sum-(Num_tyr)*(Neg_charge(pKa3_tyr,Test_pH));
        Sum:=Sum+(Num_his)*(Pos_charge(pKa3_his,Test_pH));
        Sum:=Sum+(Num_lys)*(Pos_charge(pKa3_lys,Test_pH));
        Sum:=Sum+(Num_arg)*(Pos_charge(pKa3_arg,Test_pH));
        FOR i:=1 to Num_chains DO
        BEGIN
                Temp:=(Pos_charge(N_term[i],Test_pH));
                Sum:=Sum+Temp;
                Temp:=(Neg_charge(C_term[i],Test_pH));
                Sum:=Sum-Temp;
                Temp:=Sum;
        END;
        Charge[N]:=Temp;
END;    {Find_charge}



PROCEDURE       Iterate;

VAR     Factor, Diff    :       REAL;

BEGIN
        N:=1;   Test_pH[N]:=pH1;        Find_charge;
        N:=2;   Test_pH[N]:=pH2;        Find_charge;
        REPEAT
            N:=N+1;
            BEGIN
               Factor:=((Test_pH[N-1])-(Test_pH[N-2]))/((Charge[N-1])-(Charge[N-2]));
               Test_pH[N]:=(Test_pH[N-1])-(Charge[N-1]*Factor);
               Find_charge;
               Temp:=(Charge[N])-(Charge[N-1]);
               Diff:=ABS(Temp);
            END;
        UNTIL (Diff)<=(Accuracy);
        Writeln;
        Writeln('Isoelectric point calculated to pH= ',Test_pH[N]:5:3);
        Writeln;
        Writeln('Accuracy= ',Accuracy:7:5,' pH units');
        Writeln('Number of iterations= ',N);
END;    {Iterate}



BEGIN   {main}
        Input;
        Iterate;
END.
```